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Prevalence and Expression of Genes of Type II Antitoxin Toxin Systems in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: Antibiotic resistance of bacteria has been increasing in recent years and reports indicate that some bacterial strains are even resistant to the last treatment line. The survey of MazEF antitoxin-toxin genes in 84 strain of *MRSA* and and the antimicrobial effect of supernatants on the logarithmic growth stage of the bacteria.
Methods: In this study, 84 strains of *MRSA* were collected. The patients included 48 males and 36 females with a mean age of 39 years. The primers for *Staphylococcus aureus* type II antitoxin genes were designed. In the first step, using the mecA primer and PCR, the strains were genetically examined to confirm methicillin-resistant *Staphylococcus aureus*. In the next step, the frequency of MazEF antitoxin-toxin genes was examined.
Results: All strains of methicillin-resistant *Staphylococcus aureus* had the F maz gene except one. The highest antibiotic resistance was related to the strains isolated from the wound and the lowest resistance was related to the strains isolated from the urine. the effect of the supernatant obtained

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in the death phase of *Staphylococcus aureus* was assessed and the antimicrobial effect of these supernatants on the logarithmic growth stage of the bacteria was measured.
Conclusion: since previous studies showed the antimicrobial effect of this supernatant on many other bacteria, a type II system was suspected that was confirmed by the results.

Keywords: Staphylococcus; MRSA; Antitoxin-toxin system; MazEF.

1. INTRODUCTION

Antibiotic resistance of bacteria has been increasing in recent years and reports indicate that some bacterial strains are even resistant to the last treatment line [1, 2]. This has raised many concerns in physicians, researchers, and the World Health Organization (WHO), thus calling 2019 the Year of Antibiotic Resistance and announcing that the time to seek refuge in antibiotics is coming to an end. Since then, there have been several reports announcing that bacteria will not be cured in the next few years causing many deaths worldwide, even at higher rates compared to cancer. Researchers are looking for ways to improve new treatments and discover new antibiotics [3].

Among the new therapies that have attracted the most attention are nanoparticles use, herbal remedies, combination of therapies, antimicrobial peptides, etc. However, it should be noted that the usage of these components in the clinical setting needs longer studies because of the new structure, lack of sufficient knowledge of the mechanism of action, side effects, success or failure of treatment, etc [4].

On the other hand, it is very clear that it will take a long time before a new FDA approved treatment is discovered and introduced to the market. As a result, it is important to find a treatment with a low level of resistance [5].

One of the therapeutic goals that has been highly regarded and studied by researchers in the field of antibiotic resistance is the bacterial toxin-antitoxin systems. So far more than six types of these systems have been identified. Studies have shown several functions for these systems, including bacterial survival in harsh environmental conditions, resistance to antibiotics, resistance to bacteriophages, biofilm production, and cellular apoptosis; however, scientists have not yet agreed on cellular apoptosis [6].

The most practical and common type of TA system is type II, which has been studied in many bacteria. Many studies have been carried out on *Bacillus subtillis*, and the results were promising for the effectiveness of these systems in treating antibiotic-resistant bacteria [7]. Other bacteria that have been studied include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. One of the most important advantages of type II is its high prevalence in bacteria; moreover, both toxin and antitoxin are proteins [8].

The type II system may be one of the best therapeutic goals in infections. Toxin-antitoxin systems are very important for bacterial life, and the bacteria cannot easily ignore or lose it like other genetic elements such as mobile genetic elements (mobile elements) [9].

The function of TA systems is closely related to environmental signals such as stress and food starvation, the presence of other bacteria, the presence of antibiotics, etc. In this way, each microorganism dominates unfavorable conditions using its specific and potential abilities, such as the secretion of antimicrobial substances or the expression of antimicrobial resistance gene genes [10].

On the other hand, with the indiscriminate use of antibiotics, changes in the bacterial ecological conditions, and introduction of various environmental stressors, the process of increased antibiotic resistance has reached its maximum. An important point is the transfer of these resistance factors between different bacterial strains, which has made it difficult to treat these types of infections in addition to the emergence of superbug strains in cases of simultaneous infections. Considering scientific advances in bacteriology and concerns about these conditions, it seems that it is a best and most practical way to use bacterial defense mechanisms against other bacteria [11].

The bacteria interact among them by producing chemicals. This method of communication is called chrome sensing. In addition to identifying the ways in which bacteria interact with and respond to adverse environmental conditions,

their defense mechanisms can be used to overcome these resistance mechanisms. One of the known causes of chrome sensing is a peptide called External Death Factor, which increases the expression of the toxin gene and destabilizes the antitoxin, eventually causing bacterial apoptosis [12].

Bacterial apoptosis is a clever approach to minimizing the survival of a bacterial community. This approach is associated with a lot of energy and the elimination of numerous bacteria present in a bacterial community, but the bacteria protect themselves from definite death by maintaining minimal cellular survival. Bacterial apoptosis is sometimes caused by the removal of many cells to overcome food stress, formation of biofilms to cope with stress factors such as temperature and PH changes, presence of antibacterial substances, and sometimes by the presence of secretory toxins and enzymes secreted by other bacteria. These are examples of intelligent bacterial functions [13].

Staphylococcus aureus is one of the most important bacteria in the medical field due to its tolerance and growth in various environmental conditions, possessing many pathogenic factors, having a wide range of antibiotic resistance, and causing many infections. In addition to resistance to many antibiotics, this bacterium is therefore capable of retaining resistance elements in ecology. Although resistance elements are transferred by the *enterococci*, they are preserved in the ecological cycle by *Staphylococcus aureus* [14].

The focus of the present study was on the *Staphylococcus aureus*. In previous studies, bacterial supernatant was obtained under different stress conditions with an antimicrobial potential against staphylococci and at least 10 species of other bacteria.

2. MATERIAL & METHODS

2.1 Collection and Identification of Strains

According to previous studies, all strains of *Staphylococcus aureus* carry type II toxin-antitoxin system, so the focus of sample collection has been on their distribution in important and various diseases.

The number of MRSA samples was calculated using this formula:

$$= Z^2 * P (1-P) / d^2$$

$$n = (1.96)^2 * (0.83) (0.17) / (0.08)^2 = 84$$

Л indicates the possibility of an antitoxin toxin system. So far, three type II systems have been identified for *Staphylococcus aureus*. d is the amount of accuracy that has been reduced due to the high frequency obtained in other studies.

In this study, 84 strains were collected, which can be seen in the table below. The patients included 48 males and 36 females with a mean age of 39 years.

The sources of *Staphylococcus aureus* is shown in Table 1.

Table 1. The frequency of Sources of *Staphylococcus aureus* isolation

Source	Number	Percent (%)
Tracheal	13	15.5
Wound	24	28.5
Blood	23	27.4
Urine	9	10.7
Pleural	2	2.4
Abscess	6	7.1
Synovial	2	2.4
CSF	5	5.9
Total	84	100

Staphylococcus aureus strains were collected from hospitals. Phenotypic and genotypic tests as well as the antibiotic resistance test using the Clinical and Laboratory Standards Institute (CLSI) protocol were performed in the laboratory where the project was performed.

2.2 Extraction of DNA and RNA

Staphylococcus aureus strain ATCC25923 was used as a standard strain in terms of both phenotypic and genomic antibiotic resistance. Genomic extraction was performed using the Pars Toos Kit according to the kit protocol. Immediately after RNA extraction, cDNA was prepared. The quality and quantity of the extractions and cDNA were fabricated using a nanodrop device.

The primers for *Staphylococcus aureus* type II antitoxin genes were designed. Based on bioinformatic studies in *Staphylococcus aureus* strains, all the proposed genes were selected as type II antitoxin toxin. Primers with a high percentage of specificity and sensitivity were designed for both conventional PCR and qPCR.

Primers of *Staphylococcus aureus* type II antitoxin toxin genes were designed in this study. The primers used in the design were as follows:

mazF-F:TGATTAGACGAGGAGATG
129bp=PCR pro
mazF-R: CAACAATAACTGTAGGACTAT
mazE-F:AGTCATAGCTTAGAACAA
140bp=PCR pro
mazE-R: TTCGTTGAATTAGAAGATAA

2.3 Preparation of Supernatant

This part was done in the previous study and is briefly described in the following. In the first stage, the effect of the supernatant obtained in the death phase of *Staphylococcus aureus* was assessed. Then, in addition to starvation stress, other stress conditions were used to obtain this surface material, including the presence of ciprofloxacin at a sub-MIC concentration. The antimicrobial effect of these supernatants on the logarithmic growth stage of the bacteria was measured. The fragment with the largest antibacterial effect was then isolated using rHPLC [15].

In the first step, using the mecA primer and PCR, the strains were genetically examined to confirm methicillin-resistant *Staphylococcus aureus*. In the next step, the frequency of MazEF antitoxin-toxin genes was examined. PCR products were examined on an agarose gel.

2.4 Expression Studies

The amount of cDNAs was increased to 300 ng using a Nano drop device. Expression assays were performed using a StepOnePlus™ RealTime PCR System (Applied Biosystems, Foster city, CA, USA). The RealQ Plus 2x PCR Master Mix Green without ROX™ PCR Master Mix (Parstus, Iran) was used to prepare the reactions. The mecA gene was used as normalizer.

3. RESULTS

The first step of the study was to confirm the methicillin-resistant *Staphylococcus aureus* strains among the strains sent from hospitals, which was done by determining the presence of the mec A gene in the strains. The mecA gene of PCR product is shown in Fig. 1.

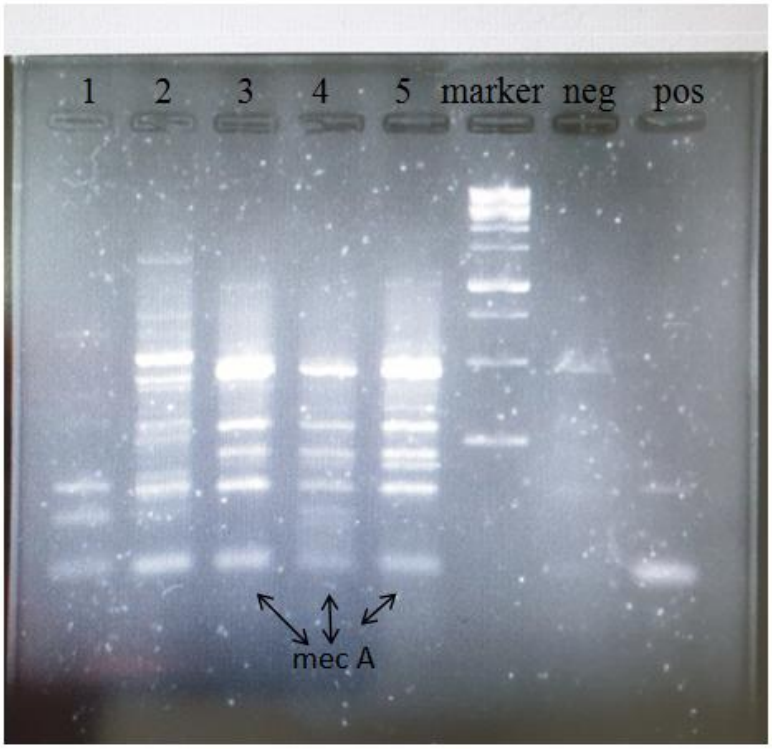

Fig. 1. Investigation of mec A gene in *Staphylococcus aureus* samples

Next, the presence of the toxin-antitoxin type II gene in the MRSA strains was investigated.

All strains of methicillin-resistant *Staphylococcus aureus* had the F maz gene except one.

The Maz F and Maz E genes of PCR product are shown in Figs. 2 & 3.

The highest antibiotic resistance was related to the strains isolated from the wound and the

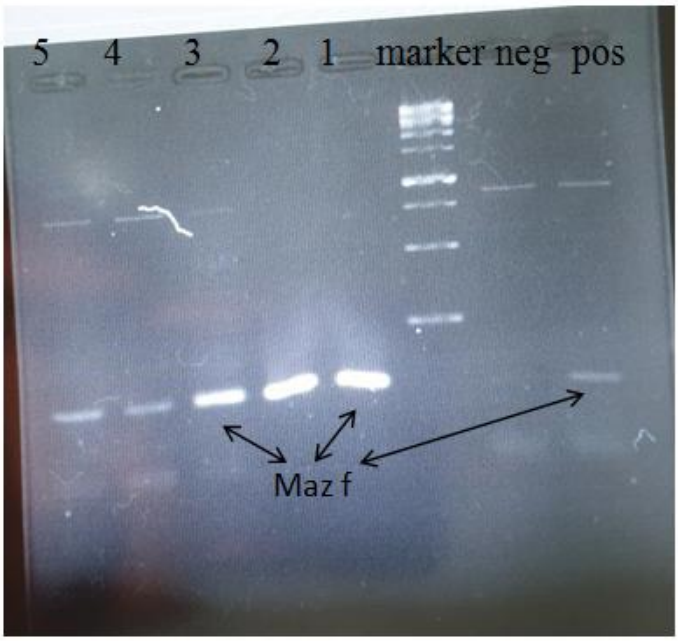

Fig. 2. Evaluation of Maz f gene in methicillin-resistant *Staphylococcus aureus* samples

Fig. 3. Evaluation of Maz E gene in methicillin-resistant *Staphylococcus aureus* samples

lowest resistance was related to the strains isolated from the urine. Because the active ingredient of the supernatant was not fully identified, the MIC was very variable in different strains isolated from the supernatant. However, in general, more pathogenic and resistant strains showed the lowest amount of MIC compared to the supernatant and vice versa. However, there were some exceptions. Of the 84 samples collected, one case did not carry the MazEF gene, which also showed no MIC relative to the supernatant. The MIC was determined by preparing solutions of the ciprofloxacine concentrations, incubating the solutions with separate batches of cultured bacteria, and measuring the results using agar dilution (Fig. 4) and broth microdilution (Fig. 5). However, in a study investigating the synergy of the supernatant and ciprofloxacin, all strains had MICs equal to the lowest amount of ciprofloxacin and the lowest amount of supernatant, even the strains resistant to ciprofloxacin. The MBC in MRSA strains relative to Ciprofloxacin (mean 0.064 mg/ml) and supernatant peptide (mean 0.4ml / 10ml culture media) is shown in Fig. 4.

As for MazEF and mecA gene expression, no significant differences were observed in any of the MIC modes with the supernatant alone and in synergism with ciprofloxacin compared to the bacterial culture without any additives. However, a significant difference was observed with respect to the recA gene such that its expression increased as the supernatant concentration increased. This phenomenon also applied to synergism. The MIC in MRSA strains relative to Ciprofloxacin (mean 0.032 mg/ml) and supernatant peptide (mean 0.2ml / 10 ml culture media) is shown in Fig. 5.

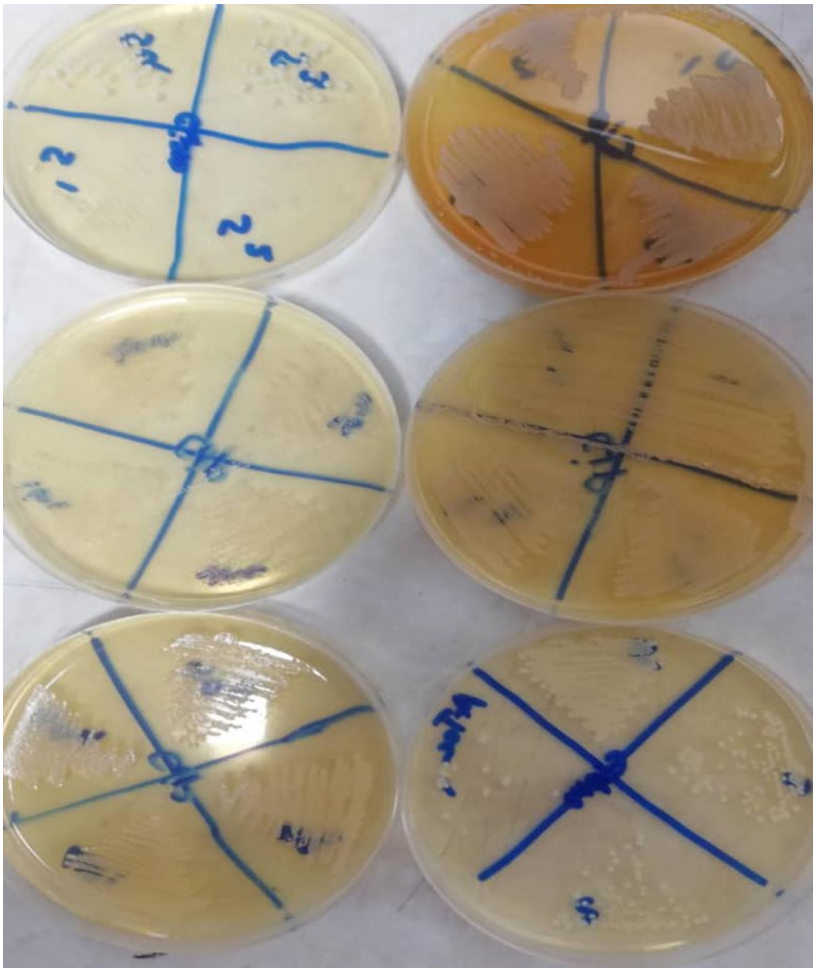

Fig. 4. Determination of MBC in MRSA samples relative to ciprofloxacin antibiotic and supernatant peptide

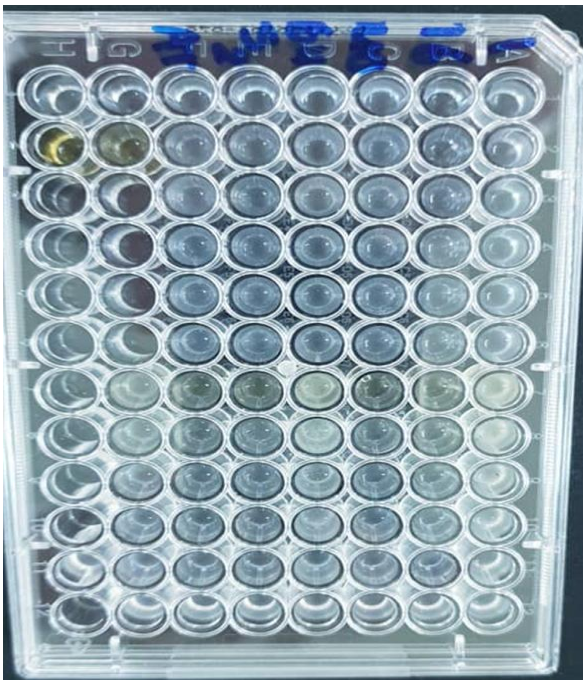

Fig. 5. Determination of MIC in MRSA samples relative to the antibiotic ciprofloxacin

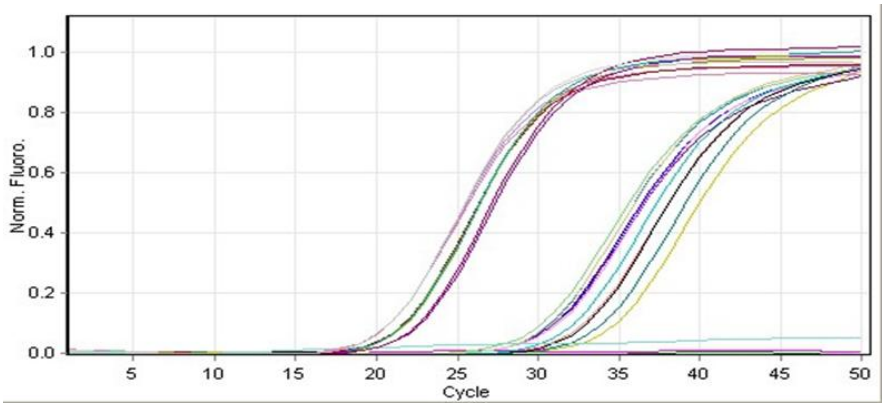

Fig. 6. Replication curves of the internal control gene and the Maz EF gene for a number of MRSA samples

Therefore, it could be concluded that the antimicrobial effect of the obtained supernatant had no significant relationship with the type II toxin system of *Staphylococcus aureus*, but this substance activated the recA gene, suggesting that the supernatant damages the bacterial genome. Replication curves of the internal control gene and the Maz EF gene for a number of MRSA samples is shown in Fig. 6.

Hence, it can be concluded that this supernatant, which resulted from bacterial culture in the BHI medium, activated the antitoxin toxin system through another mechanism or underwent changes resulting in the formation of a substance that is toxic to the bacterial genome.

4. DISCUSSION

It is interesting how the bacteria evaluate the environmental conditions and try to maintain the minimum survival of their population quite intelligently such that they plan the delay phase, growth phase, platue phase and death phase in the simplest growth conditions, i.e. on the laboratory culture media.

It has been well established that Toxin-Antitoxin (TA) systems are involved in the bacterial adaptation to their environment. Evaluation of the Quorum sensing (QS) and TA communication systems shows that in the death phase, the bacteria send a QS signal to induce this phase to counteract the death of countless cells due to the stress of food shortage and provide as much food as possible for minimal survival [16].

When *S. aureus* was exposed to oxidative stress in the phagolysosome of THP-1 macrophages, only SprF1 and SprG1 reduced, suggesting that S. aureus favors inhibiting the expression of the TA system during its internalization in the human phagocytes, which induces its death, rather than these systems that only induce stasis. This could help to promote its survival in a stressful environment [17].

Activation of TA systems when the bacteria encounter unfavorable growth conditions could explain entry into the persister state, promoting multi-drug tolerance and infection chronicity. Some bacteria enter metabolically inactive or dormant states to become persister cells, a slow-growing and drug-tolerant bacterial sub-population. In fact, the formation of persistent bacteria or stress adaptation is often mentioned as the biological roles of certain chromosomal TA systems. A period of stasis might allow the bacteria to resume normal growth, and toxins from TA systems could be involved in this process. For type I TA systems, involvement in persister cell formation via Obg in response to nutrient starvation has been demonstrated for the HokB toxin in *E. coli* [17].

Studies have shown that the quorum sensing signal secreted by the bacteria under stress conditions, known as the Extracellular Death Factor (EDF), breaks down the unstable component of the TA system, i.e. the antitoxin, and the toxin, a more stable component of the system, targets its specific sites (usually RNA cleavage) [18, 19], and causes programmed death in bacteria, a phenomenon similar to apoptosis in eukaryotic cells. This very simple and basic explanation shows the importance of these systems for bacterial survival. Thus, finding and amplifying these factors may help to discover very effective treatments for bacterial infections, even in highly resistant strains of Extensively drug-resistant (XDR) bacteria.

The most important factor considered in this study and other similar studies is signal transmission and imitation, which leads to over-activation of genetic damage repair systems in the bacteria and thus apoptosis, the mechanism that has been used by the bacteria to protect themselves over millions of years. Therefore, the cause of bacterial death is about to be discovered.

One study found that ciprofloxacin-induced stress caused the release of oxygen radicals into the environment, [20]. Another study found a peptide, which was an Extracellular Death Factor (EDF), in *Staphylococcus aureus* that caused programmed death by stimulating the type I system [21].

5. CONCLUSION

However, since previous studies showed the antimicrobial effect of this supernatant on many other bacteria, a type II system was suspected that was confirmed by the results. Such studies indicate that this supernatant has the potential to be considered as an excellent antimicrobial agent; however, further studies are needed.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

This study was approved by local conventional manner and by the ethical committee of Tehran University of Medical Sciences by number: IR.TUMS.SPH.REC.1397.093.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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